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# Plasma miR-135a; a potential biomarker for diagnosis of new type 2 diabetes (T2DM)



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## ABSTRACT

**Background:** MicroRNAs are a class of negative regulators of gene expression. Evidences indicate that miRNAs involved in the pathogenesis of New type 2 diabetes (NT2D) through decrease the expression of the genes secreting insulin and increase expression of insulin secretion suppressing ones, as well as exocytosis, incorporate in New type 2 diabetes. In this study, we evaluated the expression level of miR-135 in plasma sample of those prone to susceptible diabetes and New type 2 diabetes patients compared to the control group.

**Methods:** Subsequently to evaluation of biochemical parameters such as (TG, TC, HDL and LDL) in susceptible diabetes, New type 2 diabetes and control group, miR-135a level was measured by qRT-PCR in

the plasma samples and results were analyzed by Stata and REST softwares.

**Results:** We identified a significant increase in miR-135a expression in New type 2 diabetes and susceptible diabetes samples compared to the control group. AUC in ROC curve analysis was 1.1 respectively (confidence interval of 1.0-1.0) for NT2D and susceptible diabetes group, the best cut-off points for diagnostics in diabetics and susceptible diabetes were 2.00 and 1.02. The optimum sensitivity and specificity for both groups was 100 and 100. Results confirmed the test for 100% confidence in healthy, susceptible diabetes and New type 2 diabetes subjects.

**Conclusion:** It seems that plasma level of miR-135a can be a desirable biomarker to differentiate T2DM diabetics from the control group.

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## INTRODUCTION

Diabetes is the most prevalent endocrine disorder, and it includes several groups of metabolic disorders (such as the lack of or insulin insufficiency, and high absolute levels of glucagon) whose common feature is a hyperglycemic phenotype.<sup>1</sup> Hyperglycemia mostly occurs as a result of complex reactions between genetic, environmental, and life-style elements. However, it is also affected by factors such as insulin decrease, decrease insulin consumption, and increase insulin production.<sup>2</sup>

According to World Health Organization (WHO), the prevalence of diabetes was about 400 million people in 2013. Studies reported that this figure will increase at a fast pace and predicted to be around 600 million individuals by 2035.<sup>3</sup> There are four categories of diabetes: type 1 diabetes, type 2 diabetes, gestational diabetes, and other specific types. The most prevalent is type 2 diabetes with 85-90 percent. It is mostly observed among adults over-30 and those with obesity.<sup>1</sup>

One of the most common causes of type 2 diabetes is insulin resistance (no or decreased response to the normal levels of insulin) and abnormal secretion of insulin. Insulin resistance results from defected insulin signaling, a change in the target

protein or gene expressions, metabolic defects or interference with other hormones.<sup>4</sup>

Other factors leading to type 2 diabetes are miRNAs, a group of regulatory RNAs (~19-22 nt) that function to modulate various biological processes such as tissue development and metabolism.<sup>4,5</sup> They have fundamental roles such as in pancreas evolution; regulation of insulin secretion driven by glucose; catabolism of amino acids; formation of fatty acids in the liver; and differentiation of fat tissue, myoblast, and myogenes. Binding to 3'-UTR in one or more target mRNA, miRNAs terminates the process of translation, therefore suppressing gene expression.<sup>5</sup> Recently, evidence indicates that miRNAs are involved in the pathogenesis of T2DM through decreasing the expression of insulin-secreting genes and increasing the expression of insulin suppressing genes.<sup>6</sup>

On our previous project, we identified that the overexpression of miR-135a *in vitro* induced insulin resistance by targeting insulin signaling pathway.<sup>7</sup> Henceforth, we hypothesized that miR-135a could be an ideal blood-based biomarker for type 2 diabetes detection since miRNAs may have exceptional stability in plasma.<sup>8</sup>

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The present study aims at investigating the expression level of miR-135a in the plasma sample of those susceptible individuals and newly diagnosed type 2 diabetes patients and compare them to the control group.

## MATERIAL AND METHOD

### Subject characteristics

A total of 120 subjects were included in this case-control study. The subjects were divided into 4 groups, which were healthy individuals, people with a newly diagnosed type 2 diabetes in the last 6 months and not taking any hypoglycemic drugs, people with impaired fasting glucose (IFG), and people with impaired glucose tolerance (IGT). The study took place in the Center of Metabolic, Qazvin, Iran. The ethical code was REC.1394.191 granted from Qazvin Medical University. Informed consent was taken from all the subjects.

Subjects who had malignant tumors, cardiovascular diseases, nephropathy, and taking any hypoglycemic drugs longer than 6 months were excluded because they would have affected miRNA expressions.

### Laboratory examinations

Diabetic and pre-diabetic (IFG, IGT) subjects were confirmed with the use of WHO 1998 criteria.<sup>9</sup> The criteria were based on the serum glucose level, the fasting plasma glucose test (FPG) which were drawn after 12 hours of fasting, and oral glucose tolerance test (OGTT) test. Subjects who were confirmed by OGTT to have FPG > 7.0 mmol/L and 2 hours plasma glucose (2hPG) > 11.1 mmol/L were diagnosed as newly diagnosed type 2 diabetes patients. Meanwhile, subjects with FPG 5.6–6.9 mmol/L, and 2hPG 7.8 mmol/L were diagnosed as susceptible patients (IFG or IGT). Biochemical parameters such as triglyceride (TG), total cholesterol (TC), high-density lipoprotein (HDL), and low-density lipoprotein (LDL) were investigated in all subjects.

### RNA isolation and Quantitative Real-time PCR

Peripheral blood was placed in an EDTA containing tube and centrifuged at 2.000g for 6 minutes. After being centrifuged, the blood was aliquoted and stored at -80°C until miRNA detection.

Total RNAs were then extracted from 250 µl of plasma using TRK- 1001 (LC-Bio, USA). According to the protocol, 5 µL of plasma RNA containing miRNA was reverse transcribed to cDNA. The reverse transcription solution system consisted of 5 µl total RNA, 1 µL M-MLV Buffer, 1 µL M-MLV, 0.5 µL dNTP, and 0.5 µL RRI (Reverse Transcriptase M-MLV (RNase H-), TAKARA, CHINA). Reverse transcription reaction in Real Time PCR instrument (MyGene L96G, LongGene, CHINA). Real-time PCR was performed using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen: 11733-038). RNU6B, which was more stable in serum and rarely changed in different people, was used as an internal control gene, according to the Applied Biosystems Application Note.<sup>10,11</sup> All reactions were run in triplicate.

### Statistical analysis

Means and standard deviations of demographic and background variables in each group were calculated. We also analyzed mean and 95% CI of miR-135a. To assess the association between miR-135a and independent variables, a simple linear regression model was used. Variables with p-value <0.2, based on the R-square values, were included in a multiple-linear-regression model. We also examined multiple collinear ties and kept the most representative variable as a surrogate. To identify the pre-diabetics and T2DM, nonparametric analysis of ROC curve was used. The statistical analyses was done using the Stata software (ver. 14; College Station, Texas, USA). In order to analyze miR-135a gene expression, the REST 2009 software (Qiagen, Hilden, Germany), an independent algorithm plotting the outcome analysis for each gene as well as distributions along with the whisker box diagram, was utilized.

## RESULTS

Out of 120 subjects, 48% of the population were men and 52% were women. Means of ages ( $\pm$  SD) of the control, susceptible diabetes, and newly diagnosed type 2 diabetes group were 24.7 (2.8), 39 (38.8), and 53.8 (5.6), respectively. Findings also revealed that body mass index (BMI) was higher in newly diagnosed type 2 diabetes than other groups (Table 1).

Results from REST software indicated a significant increase in an up-regulation of miR-135a in

**Table 1** Subject characteristics

Characteristics	Control (n = 30)	Pre-Diabete (n = 60)	T2DM (n = 30)
Male	14 $\pm$ 46.4	20 $\pm$ 33.3	14 $\pm$ 46.6
BMI (kg/m <sup>2</sup> )	22.5 $\pm$ 1.9	26.8 $\pm$ 0.4	30.0 $\pm$ 1.0
Age	24.7 $\pm$ 2.8	39 $\pm$ 38.8	53.8 $\pm$ 5.6
TG	91.7 $\pm$ 6.2	126.9 $\pm$ 4.3	221.7 $\pm$ 18.9
TC	124.0 $\pm$ 5.2	214.4 $\pm$ 16.5	260.9 $\pm$ 9.4
HDL	81.6 $\pm$ 4.6	40.0 $\pm$ 31.7	30.2 $\pm$ 2.3
LDL	130.9 $\pm$ 185.0	146.1 $\pm$ 8.8	175.5 $\pm$ 7.0
Test score	0.9 $\pm$ 0.06	1.3 $\pm$ 0.2	3.6 $\pm$ 0.7

**Table 2** Mean and 95% CI of miR-135a by subjects characteristics

Characteristics	Sub Group	n of participants (%)	Mean (95% CI)*	Regression coefficients	P value
Sex	Men	48 (40.0)	1.8 (1.5 – 2.2)	1	0.659
	Women	72 (60.0)	1.8 (1.5 – 2.0)	-0.9	
Group	Control	30 (25.0)	0.9 (0.8 – 0.9)	1	< 0.001
	Susceptible	60 (50.0)	1.3 (1.3 – 1.4)	0.4	
	Newly diagnosd type 2 diabetes	30 (25.0)	3.6 (3.4 – 3.9)	2.7	
Age	Under 30	29 (24.1)	0.9 (0.8 – 0.9)	1	< 0.001
	30 – 50	73 (60.8)	1.7 (1.5 – 1.9)	0.8	
	Over 50	18 (15.0)	3.6 (3.2 – 4.0)	2.7	
BMI	Normal	29 (24.1)	0.9 (0.8 – 0.9)	1	< 0.001
	Overweight	78 (65.0)	1.8 (1.6 – 2.4)	0.9	
	Obese	13 (10.8%)?	3.9 (3.5 – 4.3)	3.0	
LDL	< 100	18 (15.0)	0.9 (0.8 – 0.9)	1	0.864
	100 – 129	11 (9.1)	0.9 (0.9 – 0.9)	0.04	
	130 – 159	56 (46.6)	1.3 (1.3 – 1.4)	0.4	
	> 160	35 (29.1)	3.3 (2.9 – 3.7)	2.4	
HDL	< 40	59 (49.1)	2.5 (2.2 – 2.8)	1	< 0.001
	40 – 50	31 (25.8)	1.3 (1.3 – 1.4)	-1.1	
	50 – 60	11 (9.1)	0.9 (0.8 – 0.9)	-1.6	
	> 60	19 (15.8)	0.9 (0.8 – 0.9)	-1.6	
TG	< 150	90 (75.0)	1.2 (1.1 – 1.2)	1	< 0.001
	150 – 199	4 (3.3)	2.8 (2.1 – 3.5)	1.6	
	200 – 500	26 (21.6)	3.8 (3.5 – 4.0)	2.6	
TC	< 200	47 (39.1)	1.0 (0.9 – 1.1)	1	0.001
	200 – 240	43 (35.8)	1.3 (1.3 – 1.4)	0.3	
	> 240	30 (25.0)	3.6 (3.4 – 3.9)	2.6	

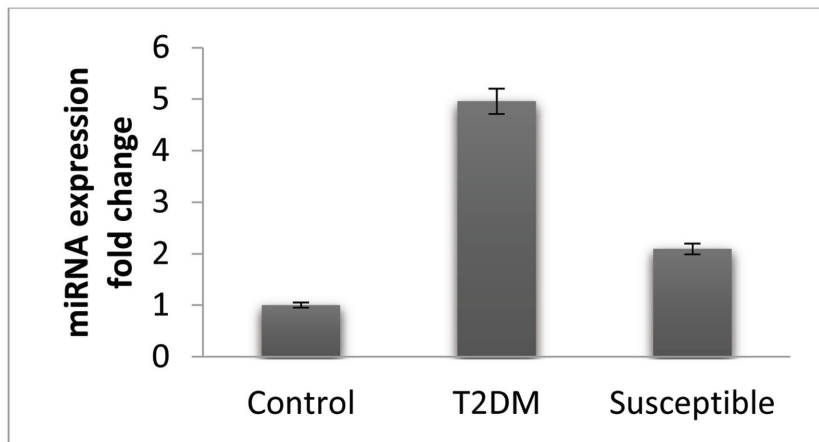
Mean of miR-135a expression

**Table 3** Multiple linear regression model to assess the association between background variables and miR-135a

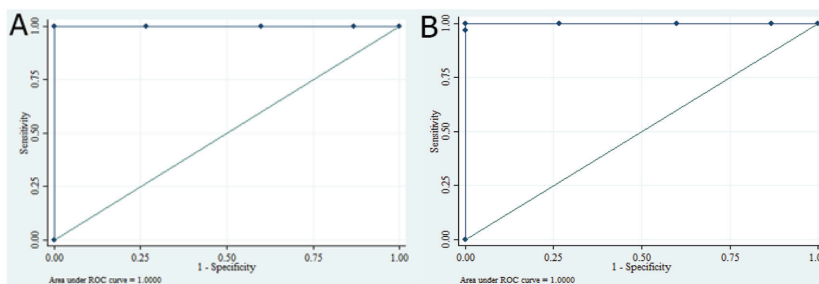
Characteristics	Sub group	Regression coefficients (95% CI)	P value
Sex	Men	1	0.477
	Women	0.05 (-0.1 – 0.2)	
Age	Under 30	1	0.855
	30 – 50	-0.07 (-0.9 – 0.7)	
	Over 50	-0.2 (-1.1 – 0.6)	
Group	Control	1	0.212
	Pre - diabetes	0.5 (-0.3 – 1.3)	
	Newly T2DM	2.9 (2.1 – 3.8)	

**Table 4** ROC curve analysis

Diseased	No of Obs	Cut-Point	Sensitivity	Specificity	Accuracy	Area ROC	CI 95%
Pre diabetes	60/30	1.02	100	100	100	1.0	1.0 – 1.0
Newly diagnosed T2DM	30/30	2.0	100	100	100	1.0	1.0 – 1.0



**Figure 1** Relative expression of miR-135a in newly T2DM and pre-diabetes compared to the control group



**Figure 2** ROC curve analysis; A: Discrimination between the cases of pre-diabetes patients and control group; B: Discrimination between the cases of newly T2DM patients and control group

newly diagnosed T2DM and pre-diabetes compared to the control group ( $p < 0.05$ ) (Figure 1).

Crude model analysis showed that there was a correlation between miR-135a expression and age, BMI, TG, TC and LDL. However, a negative correlation between miR-135a and HDL level was also observed. A significant difference between control and susceptible groups was shown, which means miR-135 may have prognostic value (Table 2).

Multivariate linear regression showed that the mean of test scores (regression coefficient) of miR-135a was 2.9 folds higher in the newly diagnosed T2DM group than the control group, after eliminating variables such as age, gender and BMI (CI 95%, 1.5-3.9). This difference was statistically significant (Table 3).

A significant difference between pre-diabetes and the control groups for miR-135a score means (Figure 1) was also found. However, we did not find any significant difference (regression coefficient=0.5; 95% CI= -0.3 – -1.3), even with the highest score means of miR-135a, after adjusting the confounding factors in the multivariate model (Table 3).

AUC in ROC curve analysis was 1.0 (95% CI 1.0-1.0) for newly diagnosed T2DM and

susceptible diabetes, the best cut-off points for diagnostics in diabetes and pre-diabetes were 2.00 and 1.02 respectively (Table 4).

The optimum sensitivity and specificity for both groups were 100 and 100. Results confirmed the test for 100% confidence in healthy, pre-diabetes (Figure 2A) and newly diagnosed T2DM subjects (Figure 2B).

## DISCUSSION

Recently, there have been a lot of investigations on the presence of miRNA in the serum and plasma as a new strong noninvasive method to diagnose cancer, cardiovascular diseases, and metabolic illnesses such as diabetes.<sup>12</sup> The first report of miRNA involvement in T2DM was published by Poy, *et al.* in 2004.<sup>13</sup> Based on the developing evidence, T2DM is associated with alterations of miRNA level in the insulin-target tissues, serum, or plasma sample. Therefore, it can be used as a predictive biomarker of diabetes.<sup>14,15</sup> In an experimental study by Yong *et al.*, miR-23a could be used as a plasma biomarker in diabetic patients.<sup>12</sup> Other studies on plasma samples of newly diagnosed diabetes also showed that miR-146a, miR-126, and miR-7 had a significant level of expression in the newly diagnosed diabetes but not in susceptible diabetes.<sup>16,17</sup> Recent findings indicate that miR-135 plays a few functional roles in the occurrence of lung cancer, gastric, colorectal, as well as Hodgkin's lymphoma syndrome.<sup>18</sup> Another study by Feng He *et al.* also reported that miR-135 involved in diabetes occurrence. It also stated that its higher expressions in diabetic nephropathy rats would lead to renal fibrosis.<sup>19</sup> In an Indian study, authors claimed that higher expression of miR-135 in musculoskeletal system of diabetic rats reduced expressions of the protein-producing insulin receptors, which was related to type-2 diabetes.<sup>20</sup>

Our results of biochemical parameters like TG, TC, HDL, LDL in newly diagnosed type 2 diabetes patients had a significant increase compared to pre-diabetes and the control group 1. These results were consistent with Kong *et al.*, who reported significant differences among biochemical parameters in the diabetics compared to the control group. However, their study showed that there was no significant difference in BMI between the two groups. Meanwhile, we found a higher BMI in newly diagnosed type 2 diabetes in crude analysis. Nonetheless, Ying Rong, *et al.* reported that newly diagnosed type 2 diabetes had higher levels of TG, TC, and BMI than the control group.<sup>17</sup>

In our prior *in vitro* project, we recognized that miR-135a overexpression in C2C12 cell line



induced insulin resistance through protein signaling involved in glucose uptake. Interestingly, our present study showed that miR-135a expression level has significantly increased in the plasma sample of newly diagnosed type 2 diabetes patients.<sup>7</sup> Agarwal *et al.* also found an increased miR-135 level in diabetic skeletal muscle and its downregulation in vivo improved glucose tolerance.<sup>21</sup> Otherwise, Honardoost *et al.* reported that in vitro induction of miR-135a had a negative effect on insulin action by targeting key components of the insulin signaling pathway.<sup>7</sup>

Abnormality of human miR-135a expression, which was shown in our present study, may have a role in reducing insulin-stimulated glucose disposal and subsequently may be a hint of a type 2 diabetes process (Table 3,  $p$ -value>0.001). Furthermore, ROC curve analysis indicated that the best cut-off points with optimum sensitivity and specificity to diagnose T2DM and pre-diabetes were 2.00 and 1.02 (Figure 2, Table 4). Consequently, evaluation of miR-135a plasma level has a valuable potential to be used as a diagnostic biomarker.

Nevertheless, based on our present data, despite the different expression level of miR-135a in pre-diabetes and control crude model, we found no statistically significant difference of the figures after adjusting the covariates (Table 3). It seems that evaluation of miR-135a as a prognostic value in pre-diabetes needs a further investigation in a larger population. In some studies, miRNA expression in pre-diabetes had no difference with the control groups.<sup>11,22-24</sup> It was partly inconsistent with our study because of the differences in samples taken either from plasma or from serum since miRNAs expressions are different in serum from plasma.<sup>25</sup>

## CONCLUSION

To sum up, plasma level of miR-135a can be a desirable biomarker to identify a newly diagnosed type 2 diabetes. However, it cannot be used in a susceptible patient. Therefore another screening method is needed to confirmed susceptible individuals.

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## CONFLICT OF INTEREST

There is no conflict of interest to be declared.

## AUTHORS' CONTRIBUTIONS

All authors had equal contribution to this article and had read and approved the final manuscript.

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